EXHIBIT A

Critical Reviews in Biotechnology, 12(4):335–356 (1992)

Gene Transfer into Mammalian Somatic Cells *In Vivo*

Ning-Sun Yang, Ph.D.
Agracetus, Inc., 8520 University Green, Middleton, WI 53562

ABSTRACT: Direct gene transfer into mammalian somatic tissues *in vivo* is a developing technology with potential application for human gene therapy. During the past 2 years, extensive progress and numerous breakthroughs have been made in this area of research. Genetically engineered retroviral vectors have been used successfully to infect live animals, effecting foreign gene expression in liver, blood vessels, and mammary tissues. Recombinant adenovirus and herpes simplex virus vectors have been utilized effectively for *in vivo* gene transfer into lung and brain tissues, respectively. Direct injection or particle bombardment of DNA has been demonstrated to provide a physical means for *in situ* gene transfer, while carrier-mediated DNA delivery techniques have been extended to target specific organs for gene expression. These technological developments in conjunction with the initiation of the NIH human gene therapy trials have marked a milestone in developing new medical treatments for various genetic diseases and cancer. Various *in vivo* gene transfer techniques should also provide new tools for basic research in molecular and developmental genetics.

KEY WORDS: gene transfer, *in vivo*, gene therapy, transfection, semantic.

I. INTRODUCTION

Gene therapy is becoming increasingly accepted as a likely approach for treatment of various and specific genetic diseases.[1-10] The current strategy for this approach is to first identify the mutant gene(s) causing a genetic defect, then to supplement the defective somatic tissues with the correct functional gene(s). Recombinant DNA technology has been used to identify and to isolate specific genes that are responsible for certain genetic diseases. Versatile technologies must now be developed to transfer functional genes into a wide variety of somatic tissues to effect gene therapy.

Gene transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transduced cells into host target organs is the main strategy for human gene therapy. Alternatively, direct transfer of functionally active foreign genes into mammalian somatic tissues or organs *in vivo* is another attractive strategy for gene therapy. During the past 2 years, a number of methods have been reported to achieve these goals with varying degrees of success. Some of these methods were obtained serendipitously. For example, direct injection of plasmid DNA into mouse muscle tissues resulted in expression of marker genes for an indefinite length of time.[11,12]

Recombinant retroviral vectors optimized for transgene expression were employed to transduce hepatocytes *ex vivo* that were later transplanted, via intrasplenic injection, into the liver of autologous host animals, effecting gene expression *in vivo*. With this method, significant levels of the low-density lipoprotein receptor gene were detected in serum of test animals.[13] Retroviral vectors have also been reported to be effective for *in vivo* and *in situ* infection of blood vessel tissues.[14] Portal vein and direct injection of retrovirus preparations into liver tissue were also shown to effect gene transfer and expression *in vivo*.[15-17] More recently, intratracheal infusion of recombinant adenovirus into lung tissues was

0738-8551/92/$.50

found to be highly effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium.[18] Herpes simplex virus vectors were also used successfully for *in vivo* gene transfer into brain tissue.[19,20]

In addition to various vector-mediated gene transfer methods, progress has also been made on direct (nonviral) gene transfer technology. We and others have demonstrated that many different types of somatic tissues can be effectively engineered *in vivo* with various marker genes constructed as plasmid DNA via a particle bombardment method.[21-23] Other techniques developed for this purpose include polylysine-mediated transfer of DNA into hepatic cells,[24,25] lipofectin- or liposome-mediated gene transfer to tissues of internal organs,[14,26,27] vesicle complexes as vehicles for gene delivery into liver,[28] electroporation of skin tissues *in vivo*,[29] and others.[11,30,31] Virtually all of these developments in gene transfer technology have the same ultimate goal: to establish and optimize techniques and procedures for future application to human gene therapy.

Besides the technological advances in gene transfer in experimental animals, even more important progress was made in the past 2 years in clinical trials of pilot human gene therapy studies. After going through extensive reviews of gene transfer and clinical treatment protocols, two challenging clinical gene therapy trials began in September 1990. In the first trial, two patients suffering from adenosine deaminase (ADA) deficiency (also known as severe combined immune deficiency (SCID) syndrome) were treated with ADA gene-transduced peripheral blood lymphocyte cells.[9,32] In another case, two melanoma patients were treated with tumor-infiltrating lymphocytes (TIL) transduced with the tumor necrosis factor (TNF) gene.[10,33] These trials, conducted by W. F. Anderson, R. M. Blaese, S. A. Rosenberg and their colleagues, were the result of many years of experimentation to improve *in vitro* gene transfer techniques in primary cultures of various lymphocyte systems. Based on their earlier studies in clinical trials using TIL cells modified to express the marker gene NPT-II for neomycin resistance (Neo)[34], they demonstrated that all tested patients tolerated the treatment well, and they observed no side effect from the gene transfer procedure per se. Although the efficacy of the present gene therapy treatments for ADA deficiency and melanoma patients has yet to be determined, an early review of the two ADA-treated patients revealed dramatic improvement.[32] These NIH clinical trials have thus set the stage for a new era of medical treatment of human diseases using gene therapy.

Only a few years ago, the concept of gene therapy for human genetic diseases, cancer, or other maladies was often viewed as a last resort. A key concern was that the tools and methods for gene delivery were limited, but today various breakthroughs involving *in vivo* and *ex vivo* gene transfer technology have been realized. This, in conjunction with the initiation of the Human Genome Project and with the increasing number of new genes identified for human diseases and cellular physiology, have confirmed the legitimacy and urgency of systematic research and development of gene therapy-related technologies.

Several new, drastically different gene transfer methods were developed very recently, almost simultaneously. Most of these methods were technically designed for specific organs. Therefore, there has been little time for adaptation or extension of specific techniques to other experimental systems, either by the group that invented the method or by other laboratories. As a result, at this stage, one cannot make direct or effective comparisons of gene transfer and expression efficiencies. This review is thus made episodic rather than systematic. An effort was also made to assess advantages and disadvantages of certain methods for future application to human gene therapy.

II. VIRUS-MEDIATED GENE TRANSFER *IN VIVO*

A. Retrovirus-Mediated Gene Transfer *Ex Vivo* and Implantation of Transduced Cells into Animals

Retrovirus vectors for mammalian gene transfer have been studied extensively during the last decade. A key development in applications of this technology to human gene therapy has been the implementation of maximal safety mea-

sures in the construction of amphotropic, replication-deficient retrovirus systems. This aspect, and other safety considerations, of using retroviral vectors for human gene therapy have been reviewed extensively by Temin.[35]

Techniques and protocols for using retroviral vectors in the transfer of genes into mammalian cells have been reviewed previously by many investigators, including Eglitis and Anderson,[36] Kohn et al.,[37] Friedmann,[38,39] Karson,[40] Kriegler,[41] Temin,[35] Miller,[42] Williams,[43] and Lo et al.[44] Continuous progress is being made on generating replication-deficient and highly "disarmed" virus vectors and amphotropic virus strains, efficient packaging cell lines, increased titers of virus preparations, and improved viral infectivity.

Using retrovirus vectors, three leading groups at the National Institutes of Health have pioneered the research on human gene therapy in various areas. Anderson, Blaese, Culver, and their co-workers[32] have introduced a normal (or correct) gene encoding adenosine deaminase (ADA) into peripheral blood lymphocytes (PBL) derived from ADA-deficient patients. These *ex vivo*, viral-transduced PBL cells were enriched in primary cultures by selection (Neo), then later injected into donor patients. Rosenberg, Anderson, Chiang, and their co-workers[33,34] have also used retroviral vectors to transfer the NPT-II gene and a potentially useful therapeutic gene (tumor necrosis factor or TNF) into tumor-infiltrating lymphocyte (TIL) cells that were isolated from tumors of melanoma patients. In January 1991, researchers transfused the *ex vivo* propagated TIL cells containing the TNF gene into two cancer donor patients, so that these cells may infiltrate the patients' tumors and release TNF to destroy cancer cells. Both of these pilot clinical trials are still in progress, so it will be some time before their efficacy can be evaluated fully.

In addition to peripheral lymphocytes and TIL cells, implantation of recombinant somatic cells back into animals, followed by expression of the introduced genes *in vivo*, has also been carried out in several other somatic cell systems. Morgan et al.[45] and Teumer et al.[46] transferred human growth hormone (hGH) genes into human kerotinocytes in culture, then grafted stably transduced epidermal cells in epithelial sheets onto athymic mice. The hGH proteins were detected in the serum of test mice at physiological concentrations for more than 4 weeks.[45]

Ponder et al.[47] isolated recombinant hepatocytes from the liver of transgenic mice and transplanted them via intrasplenic injection into recipient mice. They determined that a large fraction of these cells was localized within the liver parenchyma tissue, but not within the spleen, at 2 months after transplantation. X-gal staining assays showed that the frequency of donor cells transduced with the *E. coli LacZ* gene (encoding for β-galactosidase, β-gal) was between 0.1 and 0.3% of all hepatocytes in the recipient liver tissue, a very appreciable level. High levels of human α1-antitrypsin (α1AT, another transgenic product) were detected in the serum of transplant recipients, with expression being stable for more than 6 months. Their results suggest that transplanted hepatocytes reestablished *in vivo* may survive indefinitely. Ledley et al.[48] and Shen et al.[49] had demonstrated previously that hepatocytes in primary cultures could be transduced using retrovirus vectors, although efficiencies were relatively low in these primary cultures when compared with established cell lines. This relatively low gene transfer efficiency, however, has been improved recently by better cell manipulation techniques for primary cultures of hepatocytes (S. Woo, personal communication). Ponder et al.[50] also reported that lipofectin can be used effectively to deliver genes into primary cultures of hepatocytes, obtaining high levels of transient gene expression in transduced hepatocytes.

More recently, Chowdhury et al.[51] effectively transduced *ex vivo* rabbit hepatocytes in primary culture with recombinant retroviruses carrying a low-density lipoprotein receptor (LDLR) gene. They showed that by performing a 30% partial hepatectomy followed by intrasplenic injection of autologous, transduced hepatocytes, the great majority (95%) of the infused cells were immediately seeded into the liver via the portal circulation. They further demonstrated that upon expression of the LDLR transgene in transplanted hepatocytes, a 30 to 50% decrease in total serum cholesterol in LDLR-deficient rabbits was observed that persisted for the duration of the experiment (4 months). These results pro-

vide a good model system for the systematic exploration of liver-directed gene therapy.

Based on the results of these various groups,[47-51] a combination of more effective methods for hepatic gene transfer and an efficient procedure for transplanting transduced hepatocytes into liver tissue has been developed, which will soon be tested in human hepatic gene therapy. In mid-1991, the NIH Human Gene Therapy Subcommittee approved a proposal by Ledley, Woo, and co-workers[52] to test a gene therapy protocol for human clinical trials in acute hepatic failure and targeting marker genes to patients' hepatic cells. Later, the committee approved a similar protocol by Wilson and co-workers[51,53] to treat familial hypercholesterolemia patients, using an LDLR gene.

Salminen et al.[54] have used retroviral infection to transfer a human multidrug resistance gene (MDR1) into the genome of a rat muscle cell line (L6) and into primary rat myocytes. Transgenic myocytes were implanted into the tibialis anterior muscle of Wistar rats. Expression of MDR1 mRNA in muscles was detected at 5 d after implantation, but was minimal at 12 d. Immunosuppression of the rats with cyclosporine A resulted in detection of MDR1 mRNA at 3 to 4 weeks after implantation, a similar expression pattern to that observed for myoblasts implanted in the muscle of nude mice. These results show that implantation of recombinant myocytes into skeletal muscle may be a useful strategy for gene therapy in certain muscle diseases.

Using a recombinant retroviral vector, Dhawan et al.[55] effectively transduced a myoblast cell line (C2C12) with hGH and *LacZ* genes. After genetically engineered myoblasts were injected into mouse muscle, hGH was detected in serum for up to 3 months. Studies on cellular localization of transgenic β-gal activity showed that transduced, transplanted myoblasts effectively fused with preexisting multinucleated myofibers of target muscle tissues. Using the same approach, Barr et al.[56] reported very similar findings with C2C12 myoblast cells transduced by a calcium phosphate/plasmid DNA coprecipitation method. In this study, the levels of hGH detected in the serum of host mice were significantly lower (~4-fold less) than those observed by Dhawan et al.,[55] presumably due to lower levels of hGH expression in stably transduced myoblast cells.

B. Site-Specific Gene Transfer *In Vivo* Via Retroviral Vectors

To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific can be used. Alternatively, this can be achieved via *in situ* delivery of DNA or viral vectors to specific anatomic sites *in vivo*. Recently, several different techniques were developed for this purpose.

1. Transduction of Arterial Wall Tissues

Nabel et al.[57] first demonstrated that foreign gene transfer to blood vessels *in vivo* could be achieved by implanting *in vitro*-transduced endothelial cells in chosen sites on the arterial wall. However, since this method required cell lines to be established before gene transfer, its application to human somatic gene therapy would be limited. This concern led to the later development of a site-specific *in vivo* gene transfer method for arterial walls.

Using a recombinant retrovirus vector, Nabel et al.[14] reported a catheter delivery system that provided efficient *in vivo* retrovirus infection of specifically chosen arterial wall segments, and thereby effected long-term, site-specific gene expression in pig blood vessel tissues. To introduce either a murine amphotropic retroviral vector or DNA entrapped in liposomes, they inserted a catheter into an arterial segment. The proximal and distal balloons of the catheter were then inflated, generating a closed space into which *LacZ* transduced retrovirus was injected via the catheter device. Polybrene was injected after instillation of the virus to improve infection efficiency. X-gal chromogen assays revealed that extensive β-gal expression was readily detected for a prolonged period of time (10 d to 4 weeks) following the viral infection (Plate 1).* Maximal expression was observed between 2 and 3 months following infection. Also, *in vivo* delivery using liposomes entrapping *LacZ* DNA was found to confer β-gal

* Plates 1 and 2 appear after page 342.

expression; high levels of X-gal staining were detected after 4 d (Figure 1) and the expression persisted for more than 6 weeks.

Nabel et al.[14] have obtained additional data that show that (1) both retrovirus and liposome methods confer gene transfer and expression of marker genes in all tissue layers of the arterial wall, including the intima, media and adventia; (2) various cell types, including endothelium, vascular smooth muscle, etc., are similarly susceptible to both gene transfer methods; (3) expression via either retrovirus or liposomes is limited to the site of gene delivery and is not detectable in liver, lung, kidney or spleen; and (4) no helper virus is present in serum or tested organs.

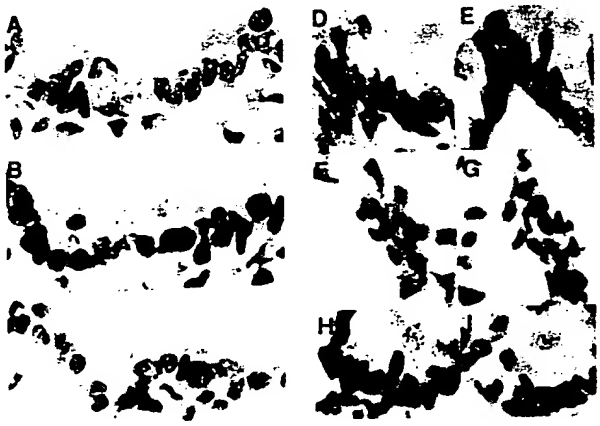

FIGURE 1. *In situ* hybridization evaluation with ^{35}S-labeled Ad-α1AT RNA probes of lung from cotton rats infected *in vivo* with Ad-α1AT by instillation into the trachea. (A) Uninfected lung (PBS control) with antisense probe. (B through I) Several examples of Ad-α1AT-infected lung. (B) Antisense probe. (C) As in (B) but with sense probe. (D) Antisense probe. (E) As in (D) but with sense probe. (F) Antisense probe. (G) As in (F) but with sense probe. (H) Antisense probe. (I) As in (H) but with sense probe. (Reprinted by permission of the publisher from Rosenfeld et al., *Science*, American Association for Advancement of Science, Washington, D.C., 1991.)

These results are important because the catheter device enables site-specific transduction of chosen blood vessel segments. Even more important, because vascular endothelial cells are exposed to blood circulation, this should be an excellent method for the release of transgenic proteins into the bloodstream, delivering secretable therapeutic proteins into the whole body. More recently, Lim et al.[58] also adopted the catheter technique for use in dogs. They showed that, by using this device to deliver the lipofectin-DNA complex, low-level firefly luciferase (Lux) gene expression was detected in arteries. Together, the findings of Nabel et al.[14] and Lim et al.[58] suggest that the balloon catheter method for gene transfer may provide a useful clinical method for treatment of human diseases such as atherosclerosis or certain cancers.

It should not be overlooked, however, that the efficiency of retroviral infection and the resulting gene expression levels are relatively low. On average, about 1% of treated cells are found to be transduced, with up to 10% transduction frequencies occasionally observed (G. Nabel, personal communication). Further optimization of the transduction protocol may result in higher levels of expression. In terms of the biology of the blood vessel tissue *in vivo*, the observed gene transfer efficiency may already be high. This is because blood vessel tissues, consisting of endothelial, smooth muscle, and other cell types, are highly differentiated and as such are not known to actively proliferate or turn over under normal physiological conditions. Since retrovirus infection and viral gene integration into the host cell genome have been shown to occur only in actively dividing cells,[59] to significantly increase *in vivo* infectivity one may have to introduce physical damage or chemical wounding to the target sites of blood vessels. Researchers also need to exercise caution in using X-gal staining for detection of transgenic β-gal activity in mammalian tissue explants, because background and β-gal-like activities can be detected among different tissue samples and may vary among different experimental animals (Yang et al., unpublished data). Flugelman et al.[60] recently showed that this concern may present a problem for X-gal staining of blood vessel tissue. Such concerns can be eliminated for some systems (either quan-

titatively or qualitatively) by using appropriate controls and enzyme staining procedures.

2. Hepatic Gene Transfer

Replication-incompetent retrovirus was used to infect livers of fetal rats by intraperitoneal injection of animals *in utero*, and to infect adult animals by direct injection into the portal vein after partial hepatectomy. With this approach, Hatzoglou et al.[15] showed that the introduced provirus sequences were integrated into the hepatic genomic DNA, with reporter gene expression detectable for up to 8 months postinfection. A liver-specific promoter from a phosphoenolpyruvate carboxykinase (PEPCK) gene was used to drive the NPT-II or bovine growth hormone (bGH) genes.

Successful viral gene expression in these experiments was obtained with the rat liver regeneration system after partial hepatectomy. Physiological and anatomical studies by Higgins and Anderson,[61] Fabrikant,[62] and Grisham[63] showed that hepatocytes can actively divide in the regenerating rat liver following the removal of up to 66% of the liver. Within the first 24 h following partial hepatectomy, synchronized DNA synthesis begins in hepatic cells followed closely by mitosis. The percentage of hepatocytes undergoing DNA synthesis increases from less than 1% to about 50% by 24 h.[62,63] Within 1 month, the liver has returned to its prehepatectomy mass, i.e., a 3- to 4-fold increase in tissue mass. Since Miller et al.[59] demonstrated that only cells that are actively dividing during infection are transduced by retrovirus, the high proliferative activity of hepatocytes in regenerating liver may thus provide an excellent environment for *in vivo* retrovirus infection. Indeed, by infecting hepatic cells of regenerating liver tissues, Hatzoglou et al.[15] demonstrated high levels (15 to 28 ng/ml/2 d) of bovine growth hormone in serum from rats infected with PbGH virus. Furthermore, they showed that the expression of the PEPCK-bGH gene was influenced by diet and hormones in a pattern similar to the endogenous regulation of the PEPCK gene in rat liver. Molecular results demonstrated the integration of foreign genes as provirus sequences in the host cell genome.

By using a different route for administration of virus particles, Kaleko et al.[16] recently reported that *in vivo* gene transfer into mouse liver can be achieved by direct injection of retrovirus preparations into liver parenchyma tissue. Instead of using partial hepatectomy to stimulate cell proliferative activity in the liver, they showed that injection of mice with an appropriate dosage of carbon tetrachloride (CCl_4), 2 d prior to surgery and virus injection, was effective in stimulating mitotic activity in liver parenchyma tissue and resulted in effective retroviral infection. PCR analysis of liver genomic DNA indicated that the presence of NPT-II gene sequences in infected liver was sustained from 10 weeks to 6 months after gene transfer, whereas livers that had not been treated with CCl_4 contained no NPT-II sequences. Two months after viral gene transfer NPT-II enzyme activity was still detectable in transduced liver tissues. These enzyme levels were very low unless the animals had been injected with azacytidine and CCl_4 prior to sacrifice.

Based on an estimate of signal intensity in their PCR analysis, Kaleko suggested that approximately 1 copy of the NPT-II gene per 160 diploid genomes was present in the transgenic liver tissues.[16] If one assumes that, on the average, one copy of the NPT-II gene is incorporated into each hepatocyte, this copy number ratio suggests that about 0.7% (1/160) of the liver's hepatocytes were transduced long term via this protocol. This presumed gene transfer rate should be verified by assaying marker gene expression at the cellular level (e.g., X-gal staining of β-gal transduced cells in liver tissue sections). This information would be very important for future applications of this or other techniques to hepatic gene therapy, because severalfold differences (e.g., 0.7 vs. 5%) in gene transfer efficiency may drastically affect the practicality of employing certain therapeutic genes against target diseases. Taking this viewpoint into consideration, Ferry et al.[17] established a surgical procedure in which regenerating rat liver (after partial hepatectomy) was temporarily excluded from the circulation system and infected *in vivo* by a 10-min asanguineous perfusion with recombinant retrovirus. Using transgenic *LacZ* with nuclear localization signals, they determined that up to 5% of the hepatocytes in the remnant liver lobes expressed

β-gal for at least 3 months after gene transfer. A 5% level of long-term transgene expression in regenerated liver tissue is quite encouraging; this technique apparently represents the most efficient method reported so far for liver-directed gene transfer.[15,16,24,51] To further evaluate hepatic gene therapy technology as a whole, it may be important to compare the various hepatic gene transfer techniques developed by Kaleko et al.,[16] Hatzoglou et al.,[15] Ferry et al.,[17] and Wu et al.[24] (to be discussed subsequently). A direct comparison within the same experimental conditions (e.g., using the *LacZ* gene driven by a strong promoter) may reveal the relative gene transfer efficiencies and the population distributions of transduced cells in target liver tissues.

3. Transduction of Mammary Tissues

In vivo gene transfer using retrovirus vectors has also been demonstrated recently to work in the rat mammary gland system. Wang et al.[64] used a thin needle to inject, via the central duct, recombinant retrovirus into the luminal space of rat mammary glands. One to 2 weeks after injection, β-gal activity was detected in about 0.3% of the total mammary epithelial cells, with expression lasting several more weeks. Southern blot analysis showed that some mammary epithelial cells had integrated the marker gene into the host cell genome, and that these cells were apparently responsible for the low but significant level of marker gene expression *in vivo*. Their results suggest that the mammary gland of breast tissues may also be employed as a target site for future gene therapy.

C. Adenovirus for *In Vivo* Gene Transfer into Lung

One limitation of the retrovirus gene delivery system is that it requires target cell proliferation for gene transfer.[59] However, most organs in adult mammals are fully developed, and most of their cells are terminally differentiated and incapable of active proliferation.[18] Therefore, under normal physiological conditions, it was believed to be difficult to transfer foreign genes *in vivo* to various mammalian somatic tissues via retroviral infection.[18] However, Nabel's[14] results in the vascular system indicate that this perception may need to be reevaluated.

To bypass this potential problem, Rosenfeld et al.[18] used a recombinant adenovirus vector to transfer a recombinant human gene to the lung respiratory epithelium *in vivo*. This approach took advantage of two past observations: " (1) host cell proliferation is not required for expression of adenovirus proteins[65,66] and (2) adenovirus are normally tropic for the respiratory epithelium." [67] In their study, the adenovirus (Ad) major late promoter was used to drive a recombinant α1AT gene and was engineered into an infectious, but replication-deficient adenovirus vector. Using this Ad vector, they successfully infected and expressed the α1AT gene in epithelial cells of the rat respiratory tract *in vivo*. After intratracheal instillation of Ad-α1AT to test rats, α1AT messenger RNA was detected in the respiratory epithelium (Figure 1), and α1AT protein was produced and secreted by infected lung tissues. High levels of α1AT were also detected in the epithelial lining fluid, lasting at least 1 week. More recently, it was found that the expression of α1AT in test animals could be extended to several months.[5,8]

Using the same experimental system, Rosenfeld et al.[68] recently extended their studies by transferring the normal human cystic fibrosis transmembrane conductance regulatory (CFTR) gene to the airway epithelium. Expression of CFTR transcripts was observed for up to 6 weeks, and the protein was detected immunologically for about 2 weeks postinfection. These results suggest that optimized *in vivo* CFTR gene transfer may provide a therapy for cystic fibrosis. It was postulated that an aerosol spray mode might be more effective than the current perfusion method in dispersing the infecting vector over a broader surface area of the intratracheal tract, resulting in a clinically convenient treatment of certain lung diseases.

The technology of using adenovirus as a vector for human gene therapy has several advantages, as summarized by Rosenfeld et al.[18]: "(1) recombination is rare, (2) there are no known associations of human malignancies with adenoviral infections despite common human infec-

tion with various adenoviruses, (3) the adenovirus genome (double-stranded piece of DNA) can be manipulated to accommodate foreign genes of up to 7.5 kb in size of foreign genes, and (4) live adenovirus has been safely used as a human vaccine." Since this adenoviral infection method for lung epithelium employs a non- or minimally invasive procedure, it is a milestone toward gene therapy of various diseases, including common genetic disorders such as α1-antitrypsin deficiency and cystic fibrosis. Also, although respiratory tract epithelium has been established as the specific and natural target site for adenoviral infection, it may be useful to determine whether the epithelial lining of other organ types (e.g., digestive tract) can also be infected using high titer of virus preparations, thus providing additional means for gene transfer into internal organs.

Another approach using adenovirus for gene transfer is being evaluated by Cotton and Curiel.[69] In their experiment, polylysine was conjugated to disarmed Ad, then foreign DNA was bound to the polylysine, forming a complex for gene delivery. Both *LacZ* and *Lux* genes were successfully transferred in this manner to various human cell types *in vitro*. The transfer of genes is successful even if the AV genome is destroyed by UV irradiation, and Cotton and Curiel suggest that "it may be possible to get rid of the viral genome altogether," mainly because foreign genes are carried "outside" the virus particle. By using the AV coat protein (and particles) only as a vehicle for internalization of the transgene construct, this approach may overcome the safety concerns of using viral gene components in therapy and the size limitation on the DNA that can be delivered. It will be important to find out if the potential advantages projected by these researchers can be confirmed in future experiments.

Adeno-associated virus (AAV) has also been evaluated as a potential vector system for human gene therapy. Samulski et al.[70] demonstrated that efficient integration of the AAV viral genome into a single, specific site on human chromosome 19 (q13.4-ter) is a key step for AAV to establish latency when helper virus is absent. This finding indicates that the AAV may provide a site-specific integration strategy for human gene therapy.

D. *In Vivo* Gene Transfer to Brain by Herpes Simplex Virus

Herpes simplex virus (HSV) is a neuronal tissue-specific virus. It can naturally persist in infected cells for a long latent period, during which only a limited number of its genes are expressed. This set of features suggests that recombinant HSV has the potential to serve as a vector for the transfer of foreign genes into the nervous system.

Recently, two research groups explored this possibility and obtained interesting results. Dobson et al.[19] used a recombinant HSV vector to inoculate mouse spinal ganglia via the sciatic nerve and hypoglossal nucleus via the tongue tissue. Deletion mutants of HSV vector were constructed to contain a *LacZ* gene driven by the mouse murine leukemia virus long terminal repeat. In whole-mount tissues, β-gal expression was shown in spinal ganglion in acute infections and in hypoglossal nucleus in latent infections.

Similar expression of β-gal in brain tissue via HSV-mediated gene transfer *in vivo* were obtained by Fink et al.[20] In this case, HSV vectors were delivered *in situ* by injection of virus preparations stereotactically into rat hippocampus and contralateral caudate. This group had previously shown that HSV mutants defective in the US3 gene exhibited an approximate 1000-fold reduction in their pathogenicity following intracranial inoculation.[71] They therefore inserted a β-gal gene driven by a viral late gene (glycoprotein C or gC) promoter so that it would disrupt the US3 gene product. With this recombinant HSV, designated US3 pgC-*LacZ*, the expression of β-gal would thus indicate the expression of foreign genes during viral replication. In another vector, d120 pLAT-*LacZ*, β-gal expression was driven by the viral latency associated transcript promoter (pLAT) and inserted into the gC locus of the deletion mutant. Expression of β-gal using this vector would thus indicate expression of foreign genes during viral latency.

This sophisticated experimental design worked as predicted. Using X-gal assays and immunocytochemistry straining of β-gal protein, they showed that β-gal expression in hippocam-

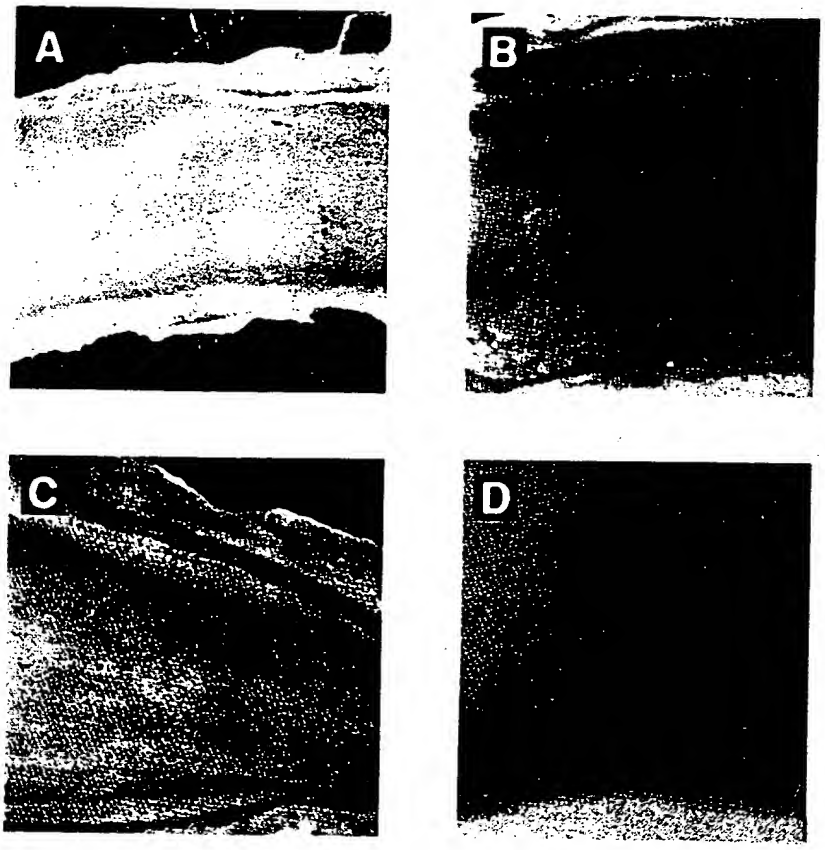

PLATE 1. Analysis of pig arterial wall infected with β-galactosidase-transducing retrovirus introduced *in vivo* by insertion of a catheter into the iliofemoral artery. β-Galactosidase activty was documented by histochemical staining in a segment of (A) normal control artery that was sham-infected or segments infected directly with the replication-defective β-galactosidase retroviral vector and analyzed after (B) 8 weeks or (C) 21 weeks, and (D) segments transduced by liposome transduction after 4 d. (Reprinted by permission of the publisher from Nabel et al., *Science*, American Association for Advancment of Science, Washington, D.C., 1990.)

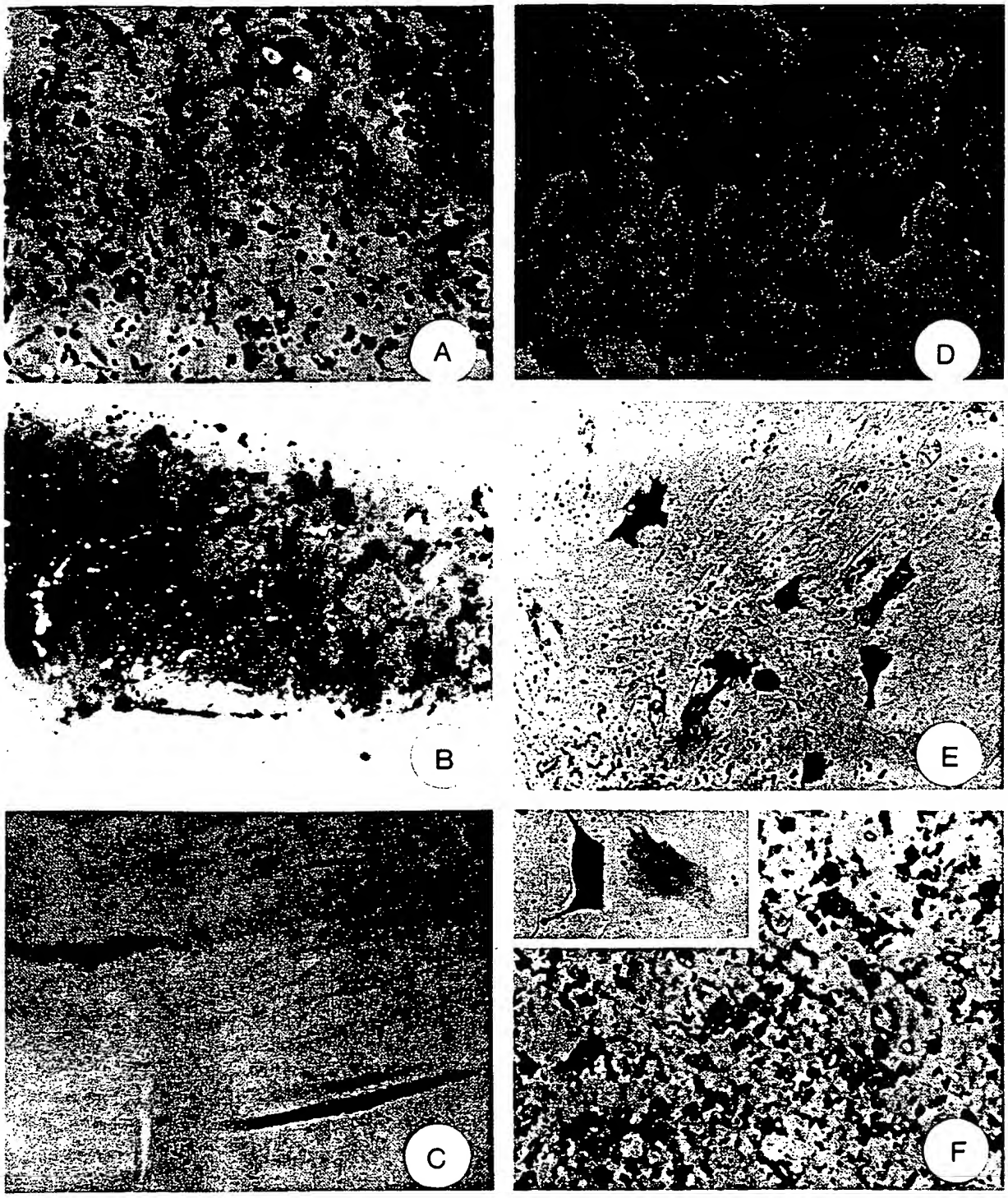

PLATE 2. Expression of CMV-β-Gal or MMTV-β-Gal activities observed at the cellular level for various bombarded tissues. Cells that are stained blue indicate β-galactosidase activity. (A) Mouse skin tissues bombarded *in vivo* with CMV-β-Gal DNA. (B) Rat liver tissues bombarded *in vivo* with CMV-β-Gal DNA. (C) Mouse muscle tissues bombarded *in vivo* with CMV-β-Gal DNA. (D) Rat mammary gland organoids bombarded *in vitro* with MMTV-β-Gal DNA. (E) Primary cultures of human mammary epithelial cells bombarded with MMTV-β-Gal DNA. (F) Human mammary carcinoma cell line (MCF-7) cells bombarded with MMTV-β-Gal DNA: Inset at high magnification. (Adapted from Yang et al., *Proc. Natl. Acad. Sci. U.S.A.,* National Academy of Sciences, Washington, D.C., 1990.)

pus was readily detected with the US3 pgC-*LacZ* vector at 2 to 3 d after HSV inoculation, with activities disappearing by day 7. Injection of the d120 pLAT-*LacZ* vector into the hippocampus did not result in focal expression of β-gal in injected tissues. Instead, isolated and scattered β-gal expressing cells were observed throughout the brain between 1 week and 3 months postinjection. Molecular analysis showed that viral genomic DNA and *LacZ* mRNA sequences were present in the brain 12 d postinjection. These results suggest that the LAT promoter was controlling β-gal expression during viral latency.[20]

The results of Fink et al.[20] and Dobson et al.[19] complement each other, supporting future experimentation of the HSV vector for brain gene therapy. Since these HSV-mediated gene transfer systems are just beginning to be explored, we may optimistically expect that future studies will generate information that will be useful not only to gene transfer technology, but also to brain research.

One major concern about human gene therapy is its safety. Although Friedmann and coworkers[72] showed that high levels of cytopathogenicity were detected when certain recombinant HSV constructs were used to transduce nerve or other cell types under *in vitro* conditions, Fink's and Dobson's *in vivo* experiments indicated that neuropathogenicity in treated brain tissues were minimal. In animals of up to 10 months postinoculation, Fink et al.[20] observed only limited disruption of the normal architecture of neuronal tissues in hippocampus. The difference in vector design and constructions used between *in vitro* and *in vivo* experiments may be a cause of the high level of cytotoxicity observed *in vitro*. Alternatively, the physiological and cellular environments of *in vivo* vs. *in vitro* brain tissues may be different enough that certain recombinant HSV vectors are intrinsically less pathogenic *in vivo* than under *in vitro* conditions. This remains to be verified in future experiments.

III. PHYSICAL MEANS FOR DIRECT GENE TRANSFER *IN VIVO*

A. Direct Gene Transfer into Muscle

Occasionally, presumed negative control samples included in experimental sets yield unexpected results. This was the case that led to the discovery of an important *in vivo* gene transfer method that is useful for muscle tissues. Wolff et al.[11] attempted gene transfer using a precipitated lipofectin-DNA complex directly injected into muscles of live experimental mice. But results obtained from injecting DNA alone were repeatedly found to have higher gene expression than those from injecting lipofectin-DNA complex. Eventually, they determined that simply by injecting a plasmid DNA solution into mouse quadriceps, high level and sustained expression of marker genes could be obtained in injected muscle tissues.[11] Gene expression level was found to be dependent upon the dosage of DNA injected. One week after DNA delivery up to 300 pg of Lux activity per target site were detected. Wolff's group initially reported that these levels were maintained for 12 weeks, but more recently they found that gene expression in some animals continued for more than 18 months, with marker gene activity in certain test animals increasing steadily throughout the experimental period (J. Wolff, personal communication). Histological studies show that clusters of striated muscle tube cells express high levels of β-gal activity.

The most intriguing finding of these muscle experiments was that the long lasting fate of gene expression was apparently not the result of foreign gene integration into the muscle cell genome. Instead, Wolff et al.[11] demonstrated by Southern blot analysis (Figure 2) that months following DNA delivery, the introduced marker genes still persisted as nonintegrated, circular plasmids that had not replicated. Therefore it was shown for the first time that certain terminally differentiated, nonproliferative tissues (e.g., muscle) could effectively maintain introduced functional genes *in vivo* in plasmid form for a prolonged period of time. Although they were not integrated into the target cell genome, these long-lasting plasmid DNA in muscle cells continued to express the functional genes they carried.

For certain gene transfer or gene therapy purposes, nonintegrative foreign gene expression may be preferable since, for example, integration-associated genomic rearrangement may lead to undesirable mutations in host genes. Also, simple injection of DNA into muscle is apparently easy to perform repeatedly. Therefore, long-term, but not necessarily permanent, transfer of therapeutic

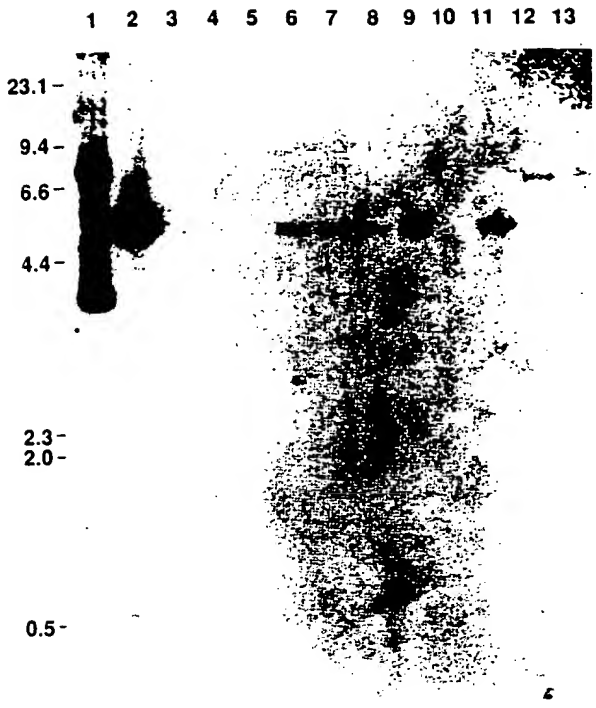

FIGURE 2. Southern Blot analysis of DNA from quadricep muscle injected 30 d previously with plasmid DNA containing RSV-Lux construct (pRSVL). Hybridization is with multiprimed ^{32}P-labeled luciferase probe from pRSVL. Lane 1, 0.05 ng of undigested pRSVL plasmid; lane 2, 0.05 ng of Bam HI-digested pRSVL; lane 3, empty; lane 4, Bam HI digest of Hirt supernatant from control muscle; lane 5, Bam HI digest of cellular DNA from control, uninjected muscle; lanes 6 and 7, Bam HI digest of Hirt supernatant from two different pools of pRSVL-injected muscles; lanes 8 and 9, Bam HI digest of cellular DNA from two different pools of pRSVL-injected muscle; lane 10, cellular DNA (as in lane 9) digested with Bam HI and Dpn I; lane 11, cellular DNA (as in lane 9) digested with Bam HI and Mbo I; lane 12, cellular DNA digested with Bgl II; and lane 13, Hirt supernatant digested with Bgl II. Size markers (/Hind III) are shown on the left in kilobases. (Reprinted by permission of the publisher from Wolff et al., *Science,* American Association for Advancement of Science, Washington, D.C., 1990.)

genes into muscle may provide a potential treatment for certain genetic deficiencies of muscle functions (e.g., Duchenne's Muscular Dystrophy). Using this method, Acsadi et al.[73] reported that low level expression of a transgenic human dystrophin gene was detectable in DNA-injected mouse muscle.

The DNA injection method for mouse quadricep muscles has been reported recently to be applicable to heart muscle tissues, but seems to be minimally effective for smooth muscle or other organ types.[12] More recently, Jiao et al.[74] showed that the direct DNA injection method seems to work best in mice, less well in rats and cats, and least in primates, suggesting potential limitations for human use. The biochemical and cellular mechanism(s) by which plasmid DNA molecules are being taken up by muscle cells *in vivo* is not clear. It is possible that transverse (T) tubules present in rough muscle fiber cells may provide the passage for DNA uptake. If so, it would explain why smooth muscle types, which do not possess T-tubules, were found to be minimally susceptible to this DNA injection method. Tracing of fluorescence labeled DNA or *in situ* hybridization at the cellular level may reveal such information in future experiments.

Another example of direct injection of DNA into tissues *in vivo* and the resulting functional expression of a foreign gene in target tissue was reported many years ago by Fung et al.[30] In this case, Rous sarcoma virus subgenomic DNA or cloned v-*src* DNA was directly injected into the wing-web of newly hatched chickens. Sarcoma tumors were generated in test birds at 3 to 4 weeks after DNA injection, with an incidence rate of $\geq$60%. Southern blot analysis showed that these tumors had acquired v-*src* sequences in the genome and expressed high levels of src messages. Both subgenomic and cloned v-*src* genes gave similar results. This study was the first to show that direct injection of cloned DNA into animal tissues *in vivo* can result in uptake, integration, and stable expression of introduced genes. Because the v-*src* gene codes for a tyrosine-specific protein kinase and is capable of transforming chicken or mouse fibroblasts, they also demonstrated that v-*src* gene alone is oncogenic *in vivo* in the avian system. Recently, England et al.[31] showed that direct injection of v-*src* DNA fragments intraperitoneally into chicken induced mesothelioma, suggesting that, at least in avian hosts, mesothelium of the peritoneal lining tissue is effective for *in vivo* uptake of naked DNA molecules.

B. Particle Bombardment-Mediated Gene Transfer

Particle-mediated gene transfer methods were first applied to the transformation of plant tissues.[75,76] With a particle bombardment device, a motive force is generated to accelerate the DNA-coated gold particles to high velocity, enabling efficient penetration of target organs, tissues, or single cells. It was thought that because this method employs a physical means for direct delivery of DNA into plant cells, either into the cytosol or into the nucleus, it could circumvent the need for interactions with cell wall for cell membrane receptors, which often exhibit cell-type specificity for vector- or chemical-mediated gene transfer methods. Indeed, later experimentation confirmed this theory.[76] One of the most important contributions of the gene-gun technology was demonstrated by Christou et al.[76] They determined that the apical meristem (the growing point tissues) of plant seedlings can be used effectively as target tissues for direct gene transfer via the particle bombardment method. As a result, chimeric plant tissues, organ segments, whole organs, and clonal plants were transformed using this gene transfer method.[76] This technology has proven to be useful for a wide variety of plant tissues and species, and has been used for the production of transgenic soybean, cotton, bean, rice, and corn (P. Christou, D. McCabe, D. Russell, and G. Brar, personal communications).

Figure 3 shows the design of an electric discharge-mediated particle acceleration device that was used initially for plant gene transfer, as reported by Christou et al.[76] It was later modified and adapted for gene transfer to various mammalian somatic tissues as described by Yang et al.[21] An important feature of the design for this device is that the particle acceleration force can be fine-tune regulated as a function of the discharge voltage. Therefore, depending on specific needs for *in vivo* target tissues or for *in vitro* cultural conditions, the penetration of gold particles can be carefully adjusted to optimize DNA delivery.

With this particle bombardment method, it was demonstrated that gene transfer to various mammalian somatic tissues can be effectively achieved in *in vivo*, *ex vivo*, and *in vitro* systems.[21-23] In the case of *in situ* bombardment of various organs of live animals, it was demon-

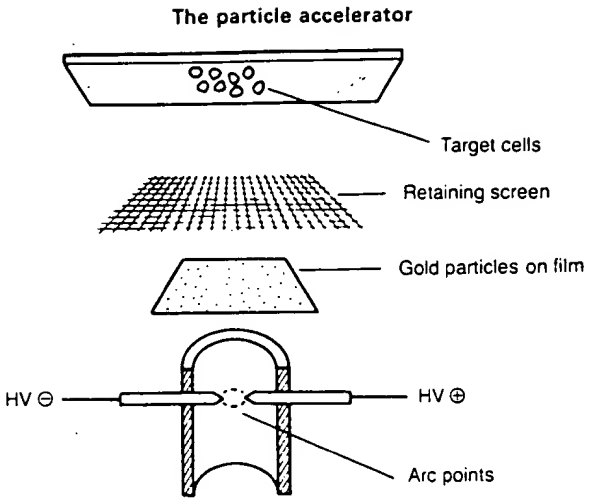

FIGURE 3. The motive force is generated in a spark discharge chamber containing two electrodes. A 10 μl water droplet is placed in between the electrodes, and a high-voltage capacitor is discharged through the water droplet that vaporizes instantly, creating a shock wave. We have found that a polyvinyl chloride pipe with an internal diameter of 13 mm is adequate for use at the spark discharge chamber. The electrodes are located opposite each other, project into the interior of the chamber approximately 5 mm below the top and are protected at the tips with an arc-resistant alloy. The gap between the two electrodes can be adjusted by appropriately threading them into or out of the spark chamber. A spacer ring is placed above the spark chamber that, in a fixed apparatus for transformations of a single crop species, can be a vertical extension of the spark discharge chamber. However, a removable spacer ring allows the distance from the spark discharge to the carrier sheet to be varied so that the force of the shock wave can be adjusted. The motive force can also be adjusted by varying the voltage of the discharge. The carrier sheet on which the DNA-coated gold particles are precipitated is placed on top of the spacer; the function of this sheet is to transfer the force of the shock wave from the spark discharge into acceleration of the carrier particles. Located above the carrier sheet is a 100-mesh stainless steel screen that retains the sheet so that it does not proceed to the target tissue. The target tissue can be placed on a water-agar plate in such a way that when the plate is inverted over the retaining screen the tissue is in the direct path of the gold particles. The whole assembly is under a partial vacuum in order to minimize aerodynamic drag. (Reprinted by permission of the publisher from Christou et al., *Trends Biotechnol.*, Elsevier, Cambridge, 1990.)

strated that particle bombardment technology can efficiently deliver foreign genes into skin and liver and muscle tissues of rat or mouse *in vivo*. Plate 2 (A to C) shows that expression of the *LacZ* gene in animals can be readily detected in targeted organs at 1 to 2 d following gene transfer. More recently, this result was extended to many other organs, including pancreas, kidney, spleen, and blood vessels (Yang et al., manuscript in preparation). For certain organs, expression of reporter genes, including *Lux* and *LacZ*, has lasted for 2 to 12 months following gene transfer, with maintenance of more than 40% of the maximal activity (detected at 1 d after bombardment). The particle-mediated gene transfer method can therefore confer long-term gene expression in certain target tissues. We have not determined whether the long-term *in vivo* gene expression observed for certain organs was a result of the integration of marker genes into the host cell nuclear genome or of the stability of plasmid DNA *in vivo*. Both seem possible, as was discussed in the previous section.

Availability of such an efficient system for *in vivo* gene transfer and transient expression, which is applicable to a variety of mammalian organs, makes it possible to assay relative strength of promoters among different organs without having to go through time-consuming germ line transgenic animal systems. Also, since the generation of transgenic animals, other than mice, is still technically very difficult, the particle gene transfer method offers the opportunity to study relative promoter strength in non-mouse, experimental animals. Toward this goal, *in vivo* promoter activity experiments, using *Lux* as a reporter gene, were performed in rats to evaluate expression of various viral and cellular promoters in different tissues (Yang et al., submitted). Two cellular promoters, phosphoglycerate kinase (PGK) and phosphoenolpyruvate carboxykinase (PEPCK) were found to exhibit interesting patterns for tissue-type expression. Results showed, for example, that *in vivo* transient expression of PGK and PEPCK promoters was 10- to 20-fold more efficient in skin than in liver or pancreatic tissues. Based on these results, it is suggested that the particle gene transfer method may be employed as a useful *in vivo* assay system for evaluating tissue specificity and relative strengths of promoters. Particle-bombardment gene transfer performed *in situ* on live animals may also offer a fast and convenient assay for the construction and the effect of therapeutic genes in target tissues of test animals.

Using particle bombardment for gene transfer, Tang et al.[77] have demonstrated that the technology also provides a method for genetic immunization. In their study, mouse ear tissue was bombarded *in vivo* to transfer plasmids containing hGH or α1AT genes. After 3 to 8 weeks, antibodies against hGH or α1AT were detected at high titer in serum of the genetically inoculated mice. This technique thus offers a simple method for eliciting a humoral immune response and it may also provide a means for genetic vaccination against pathogenic infections.

Particle bombardment technology can also be applied to *ex vivo* solid tissue explants, organ slices, or organoids.[21,23] An example is shown in Plate 2D, where freshly isolated rat mammary ductal segments (organoids) were bombarded, and expression of β-gal was readily detected at the cellular level 1 d later. Such bombarded *ex vivo* tissue materials can be monitored *in vitro* for specific experimental purposes (e.g., transgene behavior in primary cultures), or transplanted back into the body for gene therapy or other clinical purposes.

In addition to *ex vivo* and *in vivo* solid tissues, the particle bombardment method has also proven useful for *in vitro* gene transfer in cell cultures.[21,23,78,79] Various mammalian primary cultures, including those derived from the mammary gland (Plate 2E), liver and brain, were effectively bombarded to transfer a variety of marker genes, whose expressions were readily detected (Reference 20 and Yang et al., unpublished data). It is well known that many types of mammalian primary culture systems are difficult to maintain and to establish into low passage cultures. This is due to several technical difficulties: (1) surgically excised and enzymatically dissociated tissues are often obtained as cell aggregates or tissue clumps, (2) cells are difficult to remove from or reattach to culture substratum, and hence difficult to transfer into subcultures, and (3) cells are often very sensitive to changes of culture medium, treatment with chemicals, or other tissue culture manipulations. As a result, unlike established cell

culture lines, primary cell cultures can often be quite inefficient or unreliable to transduce using various gene transfer methods. Since the bombardment method involves very simple culture procedures (i.e., withdraw medium from culture, bombard cells, then add the conditioned medium back to culture — the whole procedure often takes less than 30 s to complete), it was found to be quite effective for some primary cultures.[21,23] Thus, the particle bombardment technology may prove to be a useful alternative for gene transfer to a variety of primary cell cultures. This may also have implications for some gene therapy procedures, in which cell or tissue samples from patients need to be grown out in primary cultures for gene transfer before being transplanted into patients.

Gene transfer and transient gene expression via particle bombardment has also been demonstrated in eight human cell culture lines, including cells of epithelial (Plate 2F), endothelial fibroblast, and lymphocyte origin.[21,78] Using CHO and MDF-7 cell cultures as models, stable gene transfer was obtained at frequencies of 1.7×10^{-3} and 6×10^{-4}, respectively.[21]

For potential usage in industrial or medical biosystems, the particle bombardment method may offer another practical advantage over other means of gene transfer, which is the capacity to deliver high dosage of DNA into target cells. We have determined that transient gene expression level increases at a linear rate proportional to the amount of DNA loaded onto the gold particles.[21] At a level of 1 μg of DNA per mg of gold particles, this DNA loading rate corresponds to 10,000 copies of a 5-kb DNA molecule per gold particle (1 to 3 μm). Because DNA molecules are precoated onto particles in an ethanol precipitated form, such DNA molecules on gold particles are in a dry, solid form, in contrast to being in solution. DNA-coated gold particles can deliver the predetermined and high amount of DNA into targeted cells in an effective and precise manner, for example, an average of 10,000 copies per bead for each target cell. Hence, the particle bombardment method is a practical way to achieve high-dosage DNA delivery and the resulting high expression levels.

In summary, the particle-mediated gene transfer method for delivery of foreign DNA to mammalian cells is possible for a wide range of cell types and cell environments. Since the direct physical delivery of DNA into the nucleus or cytoplasm obviously bypasses specific barriers due to membrane receptors, this method may serve as a general method for gene transfer into various mammalian somatic cells. It may provide a useful alternative to the retrovirus-based vector systems. Since it can be used *in vivo* for liver, skin, muscle, and several other organ types, many types of solid tissues or organs can now be considered target tissues for direct gene transfer. This method can also be applied *ex vivo* to various tissues, including surgically excised or biochemically dissociated organ segments or tissue clumps, as well as their derivative primary cultures. The particle bombardment procedure involves minimal manipulation of target organs, tissues, or cells and is versatile, efficient, and flexible. With further optimization of involved techniques, this technology may prove applicable to various aspects of somatic cell gene therapy.

C. Electroporation for Gene Transfer into Skin

Electroporation has been used extensively for gene transfer into a variety of cell cultures *in vitro*. Titomirov et al.[29] showed recently that it can also be applied to mouse skin tissues *in vivo*. Prior to electroporation, plasmid DNA containing the NPT-II marker gene was injected subcutaneously, thus was exposed to the dermal layer of skin tissue. Using a device that clamped electrodes onto taut skin tissue, they applied a field strength of 400 to 600 V/cm across the skin epidermal and dermal tissues, with a pulse time of about 150 μs. Twenty-four hours after electroporation the skin tissues were excised, and primary cultures of skin fibroblast cells were established. G418 selection confirmed that a significant population ($\sim 10^{-4}$) of electroporated skin cells was thus stably transduced by this method combining *in vivo* gene transfer and *in vitro* cultivation and selection. These results demonstrated that, with the appropriate system for tissue assembling, some tissues can be made susceptible to electroporation-mediated gene transfer *in vivo*. However, until significant levels of gene expres-

sion can be directly detected in live animals, it will be difficult to evaluate whether electroporation is practical for *in vivo* gene transfer.

IV. CARRIER-MEDIATED GENE TRANSFER *IN VIVO*

If therapeutic genes can be conveniently introduced into body fluids or the bloodstream, and then be directed *in vivo* to target a specific organ as the final site for functional gene expression, this route of gene transfer may have many applications for somatic gene therapy. This was the approach pursued by several laboratories, including those of Nicolau,[26] Huang,[27] and Wu.[24,25] Both liposomes and polycations (e.g., polylysine) were evaluated as carriers for *in vivo* DNA delivery.

A. Targeting Gene Transfer with Immunoliposomes

Pilot studies using liposome entrapped DNA for *in vivo* gene transfer were reported by Nicolau and co-workers.[80] They demonstrated gene expression in rat livers that had received an intravenous injection of the preproinsulin 1 gene encapsulated in conventional, pH-insensitive liposomes. Later, Soriano et al.[26] showed that liposomes bearing galactosyl groups were targeted with detectable specificity to hepatocytes *in vivo*. These results prompted other investigators to follow their examples and pursue liposome- or polylysine-mediated, target cell-specific delivery of foreign genes *in vivo*.[24,25,28]

Liposomes release their entrapped contents into the cytoplasm of target cells after they fuse with endosomal membranes.[81] To increase specific binding of liposomes to target cells *in vivo*, Wang and Huang[27] incorporated acylated monoclonal antibodies into the lipid bilayer of pH-sensitive liposomes. Their results indicated that this approach can significantly improve specificity for targeting gene expression *in vivo*. They used a chloramphenicol acetyl transferase (CAT) gene construct driven by a cAMP-regulated promoter, entrapped in H2-K^K antibody-coated liposomes. In this model system, they used RDM-4 lymphoma cells, which express the mouse major histocompatibility antigen H2-K^K, as the target cells for *in vivo* gene transfer. These cells were grown as ascites tumors in immunodeficient BALB/c mice. Then immunoliposome-entrapped DNA or free DNA were injected intraperitoneally into nude mice. At 24 h after DNA injection, about 20% of the immunoliposomes were found to be taken up by the target RDM-4 cells. Uptake was much less when control liposomes (i.e., without antibody) were used. Spleen and stomach tissues and adherent ascites cells were found to be the major sources for nonspecific uptake of liposomes.

Significant CAT activity was detected in RDM-4 cells from mice treated with pH-sensitive immunoliposomes, and the expression was found to be responsive to treatment with cAMP. CAT expression was also detected in the liver and spleen, but at much lower levels and it did not respond to cAMP treatment. It was not clear what mechanisms contributed the cAMP-independent CAT expression in these tissues. They also showed that pH-sensitive immunoliposomes were severalfold more efficient than pH-insensitive liposomes for delivering the CAT gene into the RDM-4 cells.

This study showed that pH-sensitive immunoliposomes may have applications in targeting specific tissues for *in vivo* delivery and expression of foreign genes via intraperitoneal DNA injection. However, if the uptake of liposomes by spleen and stomach tissues cannot be avoided, it may compromise this strategy for targeting gene therapy for certain diseases.

Although varying degrees of initial success with *in vivo* gene transfer via liposomes were obtained by Huang's and Nicolau's groups,[26,27] future experiments to determine the time course and relative levels of transgene expression in these liposome systems are needed.

B. Targeting Gene Transfer with Asialoglycoprotein/Polylysine

Wu et al.[24] more recently showed that marker DNA complexed to another carrier, the asialoglycoprotein/polylysine conjugate system, can target hepatocytes for *in vivo* gene transfer. Their

work was based on these previously observed findings: (1) normal hepatocytes contain unique cell surface receptors that recognize and internalize galactose-terminal (asialo) glycoproteins,[82] and (2) poly-L-lysine can bind DNA without damaging it to form strong, soluble complexes.[83] Thus, they designed a DNA delivery system targeting liver tissue by isolating orosomucoid from human serum, desialylating it to form asialoorosomucoid (ASOR), and then conjugating ASOR to polylysine (Mt = 3800). They then added this conjugate to form a complex with the plasmid DNA, which contained a CAT gene under the control of specific promoters (SV40 or albumin).

These DNA-carrier complexes were delivered into the mouse bloodstream via tail vein injection and transient CAT enzyme activity was detected in liver tissue extracts 24 h later. The maximum level was 10 units per g liver at 24 h, with activity falling to nondetectable levels by 96 h. In contrast, when partial hepatectomy was performed on the test animals, persistent levels of hepatic CAT activity were detected in the liver up to 11 weeks after the injection (Figure 4). These results[24,25] are encouraging because they show that DNA delivered as a protein complex can be efficiently targeted to liver tissues for sustained expression *in vivo*. However, because partial hepatectomy is a rather invasive surgical procedure, it may compromise the original goal of noninvasive gene therapy.

In these experiments, Wu et al.[24,25] first demonstrated the profound effect of partial hepatectomy on foreign gene expression patterns. Without partial hepatectomy, CAT activity was low, declined rapidly, and ultimately disappeared in 4d, but with partial hepatectomy, CAT activity was sustained for 11 weeks. They also reported that in some test samples the input DNA had been integrated into the host cell genome.[24] Since it was not clear what percentage of these cells were present in the regenerated liver, it cannot be determined whether integrative gene transfer alone could account for the sustained expression level. Therefore, in future experiments, it will be important to determine the extent of gene expression in liver tissues at the cellular level, especially the level of sustained gene expression. This information will also be necessary for evaluating practical applications of this procedure for clinical gene therapy of liver deficiency.

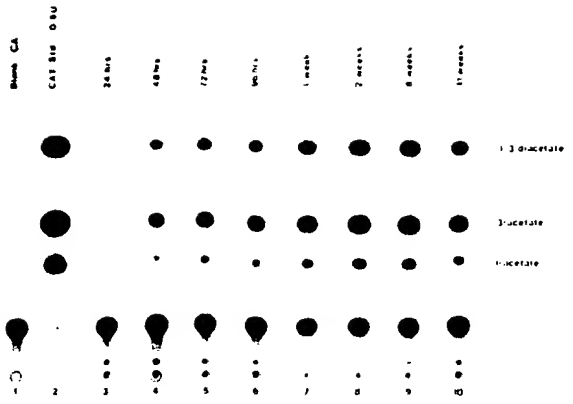

FIGURE 4. The effect of partial hepatectomy on targeted CAT gene expression. Rats were injected with AsOR-poly-L-lysine-DNA complex. A 66% partial hepatectomy was performed 30 min later, and at various time points livers were assayed for CAT activity. (Reprinted by permission of the publisher from Wu et al., *J. Biol. Chem.*, American Society for Biochemistry and Molecular Biology, Bethesda, MD, 1989.)

More recently, the same group (Wilson et al.[84]) utilized ear vein injection of the targetable DNA protein complexes to deliver a human LDLR gene into LDLR-deficient rabbits. In this study, partial hepatectomy was not performed, so the gene delivery process was not invasive. They demonstrated that high quantities of DNA can be efficiently delivered to the liver, but more than 99.9% of plasmid DNA was apparently degraded in 48 h following uptake by the liver. However, the initial high dosage of delivered DNA produced sufficient LDLR to significantly decrease the serum cholesterol level for at least 6 d following gene transfer. This noninvasive method of *in vivo* gene transfer may therefore be applicable to brief, repeated gene therapy treatments.

C. Targeting the Nucleus for Gene Transfer

Kaneda et al.[28] developed a sophisticated protocol that permits entrapment of DNA coupled with carrier nuclear proteins into specifically engineered vesicle complexes. When marker genes

were loaded into these vesicles and injected into the portal veins of adult rats, plasmid DNA was found to be efficiently carried into liver cell nuclei. Reporter genes coupled with nuclear proteins were expressed at least five times more efficiently in rat livers than the genes carried by nonnuclear proteins. Nuclear proteins that were shown to confer this effect included high-mobility group-1 protein (HMG-1, a nonhistone chromosomal protein) and nucleoplasmin (DNA binding protein extracted from *Xenopus laevis* oocytes). Bovine serum albumin was employed as the nonnuclear protein control. Marker gene expression in hepatocytes was detected at the cellular level 4 d following injection of vesicle complexes into liver.

These results suggest that nuclear proteins co-introduced into liver tissues *in vivo* can facilitate the migration of foreign DNA into the nucleus, resulting in increased levels of transient, nonintegrative gene expression at the cellular level. In the future this strategy of targeting the nucleus for gene transfer at the subcellular level may be adapted to optimize other methods of *in vivo* gene transfer.

V. RECENT PROGRESS IN *IN VITRO* GENE TRANSFER TECHNIQUES

Electroporation for gene transfer was first developed for the transduction of lymphocyte cell cultures.[85] Since then, virtually all established electroporation methods were designed for usage in tissue culture samples prepared as cell suspensions. Recently, Zheng and Chang[86] demonstrated that by using a pulsed radiofrequency electric field, high-efficiency gene transduction can be obtained with cultured mammalian cells in their attached state (*in situ*). At optimal electric field strength (~1.2 KV/cm), over 70% of the M6 test cells took up and expressed the β-gal gene, with a cell survival rate of about 80%. In contrast, less than 20% of M6 cells were transiently transduced when these cells were electroporated in suspension. Time course data suggested that the difference in gene transfer efficiency was due to the effect of the cell detachment treatment. Therefore, this new method for electroporation of cultured cells *in vitro* appears to be more efficient and convenient for some attached cells.

Chang and Reese[87] demonstrated that electroporation does indeed create pores in the cytoplasmic membranes of treated cells. Rapid-freezing electron microscopy revealed that, at 40 ms, pore-like openings were observed in cell membranes with diameters of 20 to 120 μm. These membrane pores were drastically reduced in size after 5 s, and by 10 s they had disappeared and were replaced by pit-like indentations in the membrane. Their findings provided good physical evidence that a brief (~5 to 10 s) opening of pores is most likely the mechanism responsible for gene transfer by electroporation.

Sasaki et al.[88] recently described a simple technique to introduce DNA into cells through cracks and/or pores made in cell membranes caused by intracellular ice crystal formation upon brief freezing in liquid nitrogen. Techniques and conditions were optimized for these brief freezing treatments. They showed the procedure resulted in only moderate cell killing and was effective in generating G418-resistant cell colonies transduced with a NPT-II gene as the selectable marker.

Since the methods of attached cell electroporation, brief freezing, and particle bombardment all employ a physical means for DNA delivery into cultured cells, they may circumvent cell or membrane-receptor specificity for DNA uptake. These methods may prove to be useful for gene transfer to certain cell types which are difficult to transduce by viral or liposome-mediated gene transfer methods.

Wagner et al.[89] reported recently that transferrin covalently linked to polycations (protamine or polylysine) can serve as a useful carrier for DNA uptake into erythroblasts in culture. Electrophoretically stable complexes were formed between the transferrin/polycation molecules and various DNA or RNA molecules, independent of their molecular size (up to 21 kb of DNA). Using a luciferase gene as a marker, they showed that high levels of gene expression were detected in erythroblasts, demonstrating that transferrin receptor-mediated endocytosis is efficacious for gene transfer.

VI. CONCLUDING REMARKS AND FUTURE PROSPECTS

It is obvious that within the past couple of years extensive progress has been made on *in vivo* gene transfer and expression technology. Important findings were obtained not in just one or two specific experimental systems, but in half a dozen different areas. Very diversified tools and means were established. Through these developments we are seeing the heretofore scientific and technological barriers fall away. Many scientists believe these developing technologies will have a strong and long-lasting impact on our future thinking and on the direction of gene therapy. Researchers and clinicians involved in gene transfer are being motivated by their own new findings to think further about practical and clinical applications of specific gene transfer methods. The boundary between basic science and applied technology has become blurred so that often new developments in technology have become a driving force for new directions in basic research.

Due to the fast pace of technological development, experimenters must be extremely careful in their interpretation of observations. Whereas appropriate negative controls may lead to very useful new findings (e.g., gene transfer into muscles *in vivo*), lack of appropriate control or parallel experiments can often mask the true meaning of certain observations. This may be especially true for whole animal experiments, because whole body physiology as well as the target organ anatomy can vary among different organisms, and under certain experimental conditions can vary considerably among individual animals. One cautious observation on the recent development of *in vivo* gene transfer is that, while many novel techniques are being reported, some of them are very difficult for other laboratories to adapt. Although there is understandably a high constituency of "art" in any pilot development in technology, systematic and parallel experimentation will still serve as the best approach for resolving many of these technical difficulties.

There are two key characteristics that I perceive as critical for future assessment of specific applications of gene transfer methods. First, we need to use a normalized, relatable term (i.e., specific activity) to describe gene expression levels. At present, expression levels of CAT, *LacZ*, *Lux* and other reporter genes are often being reported on a nonquantitative, arbitrary scale, which prevent comparisons between different gene transfer systems. Levels of gene expression in specific activity, expressed as the percent of transduced cells in treated cell or tissue samples, or as nanogram of transgenic protein produced per milligram of protein in tissue extract or per million test cells, can be effectively employed by other investigators to relate results to each other, avoiding ambiguity. Furthermore, these measurements, when collectively accumulated among different researchers, will serve as baseline criteria for future studies on physiological effect of various therapeutic genes. Secondly, it is also important to establish the time course of gene expression, not only for the long term, but also for the short term. Some gene transfer methodologies may provide very long-term expression, but expression levels may be low. However, such cumulative effects may be quite suitable for certain approaches of gene therapy. Conversely, an extremely high level or a burst of gene expression restricted to a short period of time may be more suitable in clinical application to some other diseases. It would thus be too early to disregard any of the currently available gene transfer methods in terms of their potential utility for gene therapy. Combinations of different methods and regimes for gene transfer could very well be profitable in certain diseases. With these considerations in mind, researchers have the opportunity and responsibility to identify the applications of various developing gene transfer methods, to optimize the techniques for specific goals, and to combine different gene transfer methods for better utility.

Although it has been minimally addressed in this review, it is apparent that there are many applications of *in vivo* gene transfer technology to various areas of basic research. It is recognized that many physiological and biochemical effects observed under *in vitro* conditions cannot be applied or demonstrated under *in vivo* conditions, and the converse may also be true. Therefore, when technically and economically possible, metabolic and physiological effects eventually have to be tested under *in vivo* conditions. *In*

vitro or *ex vivo* model systems do have their applications, but they are limited. This applies to studies on gene expression and regulation at the organ or tissue level, especially for genes that are known or expected to play a key role in physiology or metabolism. With recent advances of *in vivo* gene transfer methods, it is now possible to approach some of these experiments without having to utilize germline transgenic animal systems (which can often be time-consuming, costly, and limited to few experimental systems). Influence of foreign gene expression on growth, differentiation, and neoplastic transformation of specific tissues or organs may now be studied *in vivo* by using "transgenic tissue."

Any integrative gene transfer, other than site-specific homologous recombination, may generate mutagenic or even carcinogenic effects. The probability of this risk can be assessed with a large body of experimental evidence. The strongest argument that this probability is low is that foreign gene sequences are integrated into non-homologous sites on chromosomes in a random fashion. Also, drastic mutations of functionally important genes (including proto-oncogenes) are known to often cause cell death, eliminating these cells from the *in vivo* transduced cell population.

To minimize or eliminate these mutagenic or carcinogenic effects, researchers can adapt the homologous recombination strategy for gene transfer.[90,91] This area of research has made extensive progress recently, especially with the use of double selection methods to increase selection efficiency of correctly transduced cells.[91,92] The primary challenge in using site-specific or homologous recombination is that *in vivo* gene transfer methods must be optimized so that they will produce sufficient levels of gene expression in spite of reduced DNA recombination efficiency. Secondly, *in vivo* selection systems must be developed to eliminate random gene insertions. Increased gene delivery efficiency combined with *in vivo* selection systems would make homologous recombination practical for *in vivo* gene transfer.

In vivo technology to transfer functional genes into experimental animals is progressing at a very fast pace. Scientists and clinicians are now responsible for identifying specific clinical applications and for optimizing various gene transfer techniques to effect gene therapy. Conceived some 20 years ago by W. F. Anderson, T. Friedmann, and other pilot researchers, gene therapy is now accepted as a feasible approach for the treatment of certain genetic diseases and cancer.[93] Many researchers in this field believe that the future of gene therapy is very encouraging, though the course will certainly not be straightforward. There are still many technical problems that need to be addressed at this early stage of technology development.[94] However, with recent advances in cellular and molecular biology, tissue engineering, and gene transfer technology, many scientists and clinicians are optimistic that future breakthroughs are possible, and that they are likely to occur sooner rather than later.

ACKNOWLEDGMENTS

I deeply thank Ms. C. De Luna, P. Ziegelhoffer, and Mr. J. Burkholder for collecting information and professional editing of the manuscript. I also thank Drs. P. Christou, L. Cheng, D. McCabe, M. John, J. Haynes, K. Barton, and R. Walton for useful comments and suggestions. I am also very grateful to Ms. R. Putman for typing and final preparation of the manuscript.

REFERENCES

1. **Weatherall, D. J.,** Gene therapy in perspective, *Nature,* 349, 275, 1991.
2. **Culliton, B. J.,** Gene therapy: into the home stretch, *Science,* 249, 974, 1990.
3. **Anderson, W. F.,** Editorial: What's the rush?, *Human Gene Therapy,* 1, 109, 1990.
4. **Watson, J. D.,** Technology development in key areas can help human genome project reach its goals, *Gen. Eng. News,* 11, 4, 1991.
5. **Ezzell, C.,** Genetic therapy: just a nasal spray away?, *Science News,* 139, 246, 1991.
6. **Hoffman, M.,** New vector delivers genes to lung cells, *Science,* 252, 874, 1991.
7. **Weiss, R.,** Cancer war escalates to genetic weapons, *Science News,* 139, 69, 1991.
8. **Kotulak, R.,** Scientists find ways to give patients genes, *Chicago Tribune,* 8, Feb. 24, 1991.
9. **Thompson, L.,** Gene therapy offers hope for a host of inherited disorders, *Washington Post,* PWH9, January 1, 1991.

10. **Angier, N.,** For first time, gene therapy is tested on cancer patients, *New York Times*, January 30, 1991.
11. **Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L.,** Direct gene transfer into mouse muscle *in vivo*, *Science*, 247, 1465, 1990.
12. **Acsadi, G., Jiao, S., Jani, A., Duke, D., Williams, P., Chong, W., and Wolff, J. A.,** Direct gene transfer and expression into rat heart *in vivo*, *The New Biologist*, 3, 71, 1991.
13. **Wilson, J. M., Chowdhury, N. R., Grossman, M., Wajsman, R., Epstein, A., Mulligan, R. C., and Chowdhury, J. R.,** Temporary amelioration of hyperlipidemia in low density lipoprotein receptor-deficient rabbits transplanted with genetically modified hepatocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 87, 8437, 1990.
14. **Nabel, E. G., Plautz, G., and Nabel, G. J.,** Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall, *Science*, 249, 1285, 1990.
15. **Hatzoglou, M., Lamers, W., Bosch, F., Wynshaw-Boris, A., Clapp, D. W., and Hanson, R. W.,** Hepatic gene transfer in animals using retroviruses containing the promoter from the gene for phosphoenolpyruvate carboxykinase, *J. Biol. Chem.*, 265, 17285, 1990.
16. **Kaleko, M., Garcia, J. V., and Miller, A. D.,** Persistent gene expression after retroviral gene transfer into liver cells *in vivo*, *Human Gene Therapy*, 2, 27, 1991.
17. **Ferry, N., Duplessis, O., Houssin, D., Danos, O., and Heard, J.-M.,** Retroviral-mediated gene transfer into hepatocytes *in vivo*, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 8377, 1991.
18. **Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Paakko, P. K., Giraldi, P., Stratford-Perricaudet, L. D., Perricaudet, M., Jallat, S., Pavirani, A., Iecocq, J., and Crystal, R. G.,** Adenovirus-mediated transfer of a recombinant α-1-antitrypsin gene to the lung epithelium *in vivo*, *Science*, 252, 431, 1991.
19. **Dobson, A. T., Margolis, T. P., Sedarati, F., Stevens, J. G., and Feldman, L. T.,** A latent nonpathogenic HSV-1-derived vector stably expresses β-galactosidase in mouse neurons, in *Miami Short Reports-Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol. 1, Ahmad, F., Bialy, H., Black, S., Howell, R. R., Johnson, D. H., Lubs, H. A., Puett, J. D., Rabin, M. B., Scott, W. A., Brunt, J. V., and Whelan, W. J., Eds., Boehringer Mannheim Biochemicals, USA, 1991, 43.
20. **Fink, D. J., Sternberg, L. R., Mata, M., Goins, W., and Glorioso, J. C.,** *In vivo* expression of beta-galactosidase in brain by HSV-mediated gene transfer in *Miami Short Reports-Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol. 1, Ahmad, F., Bialy, H., Black, S., Howell, R. R., Johnson, D. H., Lubs, H. A., Puett, J. D., Rabin, M. B., Scott, W. A., Brunt, J. V., and Whelan, W. J., Eds., Boehringer Mannheim Biochemicals, USA, 1991, 43.
21. **Yang, N.-S., Burkholder, J., Roberts, B., Martinell, B., and McCabe, D.,** *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment, *Proc. Natl. Acad. Sci. U.S.A.*, 87, 9568, 1990.
22. **Williams, R. S., Johnston, S. A., Riedy, M., DeVit, M. J., McElligott, S. G., and Sanford, J. C.,** Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 2726, 1991.
23. **Zelenin, A. V., Alimov, A. A., Titomirov, A. V., Kazansky, A. V., Goredetsky, S. I., and Kolesnikov, V. A.,** High-velocity mechanical DNA transfer of the chloramphenicolacetyl transferase gene into rodent liver, kidney and mammary gland cells in organ explants and *in vivo*, *FEBS Lett.*, 280, 94, 1991.
24. **Wu, C. H., Wilson, J. M., and Wu, G. Y.,** Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements *in vivo*, *J. Biol. Chem.*, 264, 16985, 1989.
25. **Wu, G. Y. and Wu, C. H.,** Receptor-mediated gene delivery and expression *in vivo*, *J. Biol. Chem.*, 263, 14621, 1988.
26. **Soriano, P., Dijkstra, J., Legrand, A., Spenjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G., and Nicolau, C.,** Targeted and nontargeted liposomes for *in vivo* transfer to rat liver cells of a plasmid containing the preproinsulin I gene, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 7128, 1983.
27. **Wang, C.-Y. and Huang, L.,** pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 7851, 1987.
28. **Kaneda, Y., Iwai, K., and Uchida, T.,** Increased expression of DNA cointroduced with nuclear protein in adult rat liver, *Science*, 243, 375, 1989.
29. **Titomirov, A. V., Sukharev, S., and Kistanova, E.,** *In vivo* electroporation and stable transformation of skin cells of newborn mice by plasmid DNA, *Biochim. Biophys. Acta*, 1088, 131, 1991.
30. **Fung, Y.-K. T., Crittenden, L. B., Fadly, A. M., and Kung, H.-J.,** Tumor induction by direct injection of cloned v-*src* DNA into chickens, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 353, 1983.
31. **England, J. M., Panella, M. J., Ewert, D. L., and Halpern, M. S.,** Induction of a diffuse mesothelioma in chickens by intraperitoneal inoculation of v-*src* DNA, *Virology*, 182, 423, 1991.
32. **Gorber, P.,** Early gene therapy gets rave reviews, *Chicago Tribune*, PCI, July 28, 1991.
33. **Chiang, Y. W., Friedmann, S., Hammer, M., Yang, S., Wadhams, K., Kuebbing, D., Tolstoshev, P., and Anderson, F.,** Lymphokine gene expression using retroviral vectors, *J. Cell. Biochem. Suppl.*, 15A, 186, 1991.

34. **Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R. A., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., Merino, M. J., Culver, K., Miller, A. D., Blaese, R. M., and Anderson, W. F.,** Gene transfer into humans — immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction, *N. Engl. J. Med.*, 323, 570, 1990.
35. **Temin, H. M.**, Safety considerations in somatic gene therapy of human disease with retrovirus vectors, *Human Gene Therapy*, 1, 111, 1990.
36. **Eglitis, M. A. and Anderson, W. F.,** Retroviral vectors for introduction of genes into mammalian cells, *BioTechniques*, 6, 608, 1988.
37. **Kohn, D. B., Anderson, W. F., and Blaese, R. M.,** Gene therapy for genetic diseases, *Cancer Invest.*, 7, 179, 1989.
38. **Friedmann, T., Xu, L., Wolff, J., Yee, J. K., and Miyanohara, A.,** Retrovirus vector-mediated gene transfer into hepatocytes, *Mol. Biol. Med.*, 6, 117, 1989.
39. **Friedmann, T.,** Progress toward human gene therapy, *Science*, 244, 1275, 1989.
40. **Karson, E. M.,** Prospects for gene therapy, *Biol. Reproduction*, 42, 39, 1990.
41. **Kriegler, M.,** *Gene Transfer and Expression: A Laboratory Manual*, Stockton Press, New York, 1990, 96–102, 161–163.
42. **Miller, A. D.,** Retrovirus packaging cells, *Human Gene Therapy*, 1, 5, 1990.
43. **Williams, D. A.,** Expression of introduced genetic sequences in hematopoietic cells following retroviral-mediated gene transfer, *Human Gene Therapy*, 1, 229, 1990.
44. **Lo, M. M., Conrad, M. K., Mamalaki, C., and Kadan, M. J.,** Retroviral-mediated gene transfer. Applications in neurobiology, *Mol. Neurobiol.*, 2, 155, 1988.
45. **Morgan, J. R., Barrandon, Y., Green, H., and Mulligan, R. C.,** Expression of an exogenous growth hormone gene be transplantable human epidermal cells, *Science*, 237, 1476, 1987.
46. **Teumer, J., Lindahl, A., and Green, H.,** Human growth hormone in the blood of athymic mice grafted with cultures of hormone-secreting human keratinocytes, *FASEB J.*, 4, 3245, 1990.
47. **Ponder, K. P., Gupta, S., Leland, F., Darlington, G., Finegold, M., DeMayo, J., Ledley, F. D., Chowdhury, J. R., and Woo, S. L.,** Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 1217, 1991.
48. **Ledley, F. D., Darlington, G. J., Hahn, T., and Woo, S. L. C.,** Retroviral gene transfer into primary hepatocytes: implications for genetic therapy of liver-specific functions, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 5335, 1987.
49. **Shen, R.-F., Clift, S. M., DeMayo, J. L., Sifers, R. N., Finegold, M. J., and Woo, S. L. C.,** Tissue-specific regulation of human alpha 1-antitrypsin gene expression in transgenic mice, *DNA*, 8, 101, 1989.
50. **Ponder, K. P., Dunbar, R. P., Wilson, D. R., Darlington, G. J., and Woo, S. L. C.,** Evaluation of relative promoter strength in primary hepatocytes using optimized lipofectin, *Human Gene Therapy*, 2, 41, 1991.
51. **Chowdhury, J. R., Grossman, M., Gupta, S., Chowdhury, N. R., Baker, J. R., and Wilson, J. M.,** Long-term improvement of hypercholesterolemia after *ex vivo* gene therapy in LDLR-deficient rabbits, *Science*, 254, 1802, 1991.
52. **Ledley, F. D., Woo, S. L. C., Ferry, G. D., Whisennand, H. H., Brandt, M. L., Darlington, G. J., Demmler, G. J., Finegold, M. J., Pokorny, W. J., Rosenblatt, H., Schwartz, P., Anderson, W. F., and Moen, R. C.,** Hepatocellular transplantation in acute hepatic failure and targeting genetic markers to hepatic cells, *Human Gene Therapy*, 2, 331, 1991.
53. **Ezzell, C.,** Gene therapy meets liver transplants, *Science News*, 139, 228, 1991.
54. **Salminen, A., Elson, H. F., Mickley, L. A., Fojo, A. T., and Gottesman, M. M.,** Implantation of recombinant rat myocytes into adult skeletal muscle: a potential gene therapy, *Human Gene Therapy*, 2, 15, 1991.
55. **Dhawan, J., Pan, L. C., Pavlath, G. K., Travis, M. A., Lanctot, A. M., and Blau, H. M.,** Systemic delivery of human growth hormone by injection of genetically engineered myoblasts, *Science*, 254, 1509, 1991.
56. **Barr, E. and Leiden, J. M.,** Systemic delivery of recombinant proteins by genetically modified myoblasts, *Science*, 254, 1507, 1991.
57. **Nabel, E. G., Plautz, F. M., Boyce, J. C., Stanley, G. J., and Nabel, G. J.,** Recombinant gene expression *in vivo* within endothelial cells of the arterial wall, *Science*, 244, 1342, 1989.
58. **Lim, C. S., Chapman, G. D., Gammon, R. S., Muhlestein, J. B., Bauman, R. P., Stack, R. S., and Swain, J. L.,** Direct *in vivo* gene transfer into the coronary and peripheral vasculatures of the intact dog, *Circulation*, 83, 2007, 1991.
59. **Miller, D. G., Adam, M. A., and Miller, A. D.,** Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection, *Mol. Cell Biol.*, 10, 4239, 1990.
60. **Flugelman, M. Y., Jaklitsch, M. T., Newman, K. D., Casscells, W., Bratthauer, G. L., and Dichek, D. A.,** Low level *in vivo* gene transfer into the arterial wall through a perforated balloon catheter, *Circulation*, 85, 1110, 1992.
61. **Higgins, G. M. and Anderson, R. M.,** Experimental pathology of the liver, *Arch. Pathol.*, 12, 186, 1931.

62. **Fabrikant, J. I.,** The kinetics of cellular proliferation in regenerating liver, *J. Cell Biol.*, 36, 551, 1968.
63. **Grisham, J. W.,** A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3, *Cancer Res.*, 22, 842, 1962.
64. **Wang, B., Kennan, W. S., Yasukawa-Barnes, J., Lindstrom, M. J., and Gould, M. N.,** Carcinoma induction following direct *in situ* transfer of v-Ha-*ras* into rat mammary epithelial cells using replication-defective retrovirus vectors, *Cancer Res.*, 51, 2642, 1991.
65. **Horowitz, M. S.,** Adenoviridae and their replication, in *Virology*, Fields, B. N. and Knipe, D. M., Eds., Raven, New York, 1990, 1679.
66. **Berkner, K. L.,** Development of adenovirus vectors for the expression of heterologous genes, *Biotechniques*, 6, 616, 1988.
67. **Strauss, S. E.,** Adenovirus infections in humans, in *The Adenoviruses*, Ginsberg, H. S., Ed., Plenum, New York, 1984, chap. 11.
68. **Rosenfeld, M. A., Yoshimura, K., Trapnell, B. C., Yoneyama, K., Rosenthal, E. R., Dalemans, W., Fukayama, M., Bargon, J., Stier, L. E., Stratford-Perricaudet, L., Perricaudet, M., Guggino, W. B., Pavirani, A., Lecocq, J.-P., and Crystal, R. G.,** *In Vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium, *Cell*, 68, 143, 1992.
69. **Fox, J. L.,** Moving human genes, *Biotechnology*, 10, 17, 1992.
70. **Samulski, R. J., Zhu, X., Xiao, X., Brook, J. D., Housman, D. E., Epstein, N., and Hunter, L. A.,** Targeted integration of adeno-associated virus (AAV) into human chromosome 19, *EMBO J.*, 10, 3941, 1991.
71. **Weber, P. C., Levine, M., and Glorioso, J. C.,** Rapid identification of nonessential genes of herpes simplex virus type I by Tn5 mutagenesis, *Science*, 236, 576, 1987.
72. **Johnson, P. A., Roemer, K. B., Yoshida, K., Gage, F. H., Roman, M., Miyanohara, A., and Friedmann, T.,** Efficient gene transfer with herpes simplex-based vectors, *J. Cell. Biochem. Suppl.*, 15A, 172, 1991.
73. **Acsadi, G., Dickson, G., Love, D. R., Jani, A., Walsh, F. S., Gurusinghe, A., Wolff, J. A., and Davies, K. E.,** Human dystrophin expression in MDX mice after intramuscular injection of DNA constructs, *Nature*, 352, 815, 1991.
74. **Jiao, S., Williams, P., Berg, R. K., Hodgeman, B. A., Liu, L., Repetto, G., and Wolff, J. A.,** Direct gene transfer into nonhuman primate myofibers *in vivo*, *Human Gene Therapy*, 3, 21, 1992.
75. **Klein, T. M., Wolf, E. D., Wu, R., and Sanford, J. C.,** High-velocity microprojectiles for delivering nucleic acids into living cells, *Nature*, 327, 70, 1987.
76. **Christou, P., McCabe, D. E., Martinell, J., and Swain, W. F.,** Soybean genetic engineering-commercial production of transgenic plants, *Trends Biotechnol.*, 6, 145, 1990.
77. **Tang, D.-C., DeVit, M., and Johnston, S. A.,** Genetic immunization is a simple method for eliciting an immune response, *Nature*, 356, 152, 1992.
78. **Zelenin, A. V., Titomirov, A. V., and Kolesnikov, V. A.,** Genetic transformation of mouse cultured cell with the help of high-velocity mechanical DNA injection, *FEBS Lett.*, 244, 65, 1989.
79. **Johnston, S. A., Riedy, M., DeVit, M. J., Sanford, J. C., McElligott, S., and Williams, R. S.,** Biolistic transformation of animals tissue, *In Vitro Cell. Dev. Biol.*, 27P, 11, 1991.
80. **Nicolau, C., Le Pape, A., Soriano, P., Fargette, F., and Juhel, M.-F.,** *In vivo* expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 1068, 1983.
81. **Huang, L., Connor, J., and Wang, C. Y.,** pH-sensitive immunoliposomes, *Methods Enzymol.*, 149, 88, 1987.
82. **Ashwell, G. and Morell, A. G.,** The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins, *Adv. Enzymol.*, 41, 99, 1974.
83. **Chang, C., Weiskopf, M., and Li, H. J.,** Conformational studies of nucleoprotein. Circular dichroism of deoxyribonucleic acid base pairs bound by polylysine, *Biochemistry*, 12, 3028, 1973.
84. **Wilson, J. M., Grossman, M., Wu, C. H., Chowdhury, N. R., Wu, G. Y., and Chowdhury, J. R.,** Hepatocyte-directed gene transfer *in vivo* leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits, *J. Biol. Chem.*, 267, 963, 1992.
85. **Potter, H., Weir, L., and Leder, P.,** Enhancer-dependent expression of human k immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 7161, 1984.
86. **Zheng, Q. and Chang, D. C.,** High-efficiency gene transfection by in situ electroporation of cultured cells, *Biochimica et Biophysica Acta*, 1088, 104, 1991.
87. **Chang, D. C. and Reese, T. S.,** Changes in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy, *Biophys. J.*, 58, 1, 1990.
88. **Sasaki, K., Mizusawa, H., Ishidate, M., and Tanaka, N.,** Gene transfer into mammalian cells by rapid freezing, *In Vitro Cell. Dev. Biol.*, 27A, 86, 1991.
89. **Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L.,** Transferrin-polycation conjugates as carriers for DNA uptake into cells, *Proc. Natl. Acad. Sci. U.S.A.*, 87, 3410, 1990.

90. **Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., and Smithies, O.,** Targeted correction of a mutant HPRT gene in mouse embryonic stem cells, *Nature*, 330, 576, 1987.

91. **Mansour, S. L., Thomas, K. R., and Capecchi, M. R.,** Disruption of the proto-oncogene *int*-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to nonselectable genes, *Nature*, 336, 348, 1988.

92. **Mansour, S. L., Thomas, K. R., Deng, C., and Capecchi, M. R.,** Introduction of a *lacZ* reporter gene into the mouse *int*-2 locus by homologous recombination, *Proc. Natl. Acad. Sci. U.S.A.*, 87, 7688, 1990.

93. **Culliton, B. J.,** Gene therapy on the move, *Nature*, 354, 429, 1991.

94. **Kolberg, R.,** Gene-transfer virus contaminant linked to monkey cancer, *J. NIH Res.*, 4, 43, 1992.